BIOMACROMOLECULE POLYMER CONJUGATES

[0001] This application claims benefit of US Provisional Application 60/511,752 filed October 15, 2003. This invention relates to the formation of protein-polymer conjugates which have therapeutic uses, are intermediates for forming other materials or usable in diagnostic sensors.

BACKGROUND

[0002] Complexes between synthetic polymers and biological macromolecules can provide important commercial therapeutics as well as valuable building blocks of structured materials and sensors. Conjugation of therapeutic proteins with polymers, for example with polyethylene glycol, has been shown to prolong the serum half-life and reduce immunogenicity of the proteins. Conjugates of synthetic polymers covalently linked to biomacromolecules at the ends of the biomacromolecule are examples of such complexes. In this instance, controlling the site of covalent conjugation, the number of polymer chains conjugated, and the length, dispersity, and architecture of the synthetic polymer chains are particularly critical to resultant properties.

[0003] The currently used method to prepare covalent biomacromolecule-polymer complexes involves first preparing a polymer chain modified with a reactive end group and subsequently conjugating that preformed polymer to the biomacromolecule which contains either a natural or a non-natural amino acid. Kiick, K.L. et al. ("Expanding the Scope of Protein Biosynthesis by Altering the Methionyl-tRNA Synthetase Activity of a Bacterial Expression Host", Angew. Chem., Int. Ed., 39 (2000) p2148-2152; "Identification of an Expanded Set of Translationally Active Methionine Analogues in Escherichia Coli", FEBS Lett., 502 (2001) p25-30) discusses the incorporation of non-natural amino acids into proteins.

[0004] US Patent 5,998,588 to Hoffman et al. is an example of several patents issued to Hoffman covering various procedures for the conjugation of preformed polymer chains to numerous biomolecules including proteins.

[0005] Kochendoerfer, G.G. et al. ("Design and Chemical Synthesis of a Homogeneous Polymer-Modified Erythropoiesis Protein", Science, 299 (2003) p884-887) gives an example of the formation of a polymer modified protein by chemical synthesis, an amino-

oxy group on the polymer being linked at ketone bearing Lys (N-levulinyl) residues on the peptide.

[0006] Wang Y. et al. ("Structural and Biological Characterization of Pegylated Recombinant Interferon Alpha-2b and its Therapeutic Implications", Adv. Drug Delivery Rev., 54 (2002) p547-570) discusses the therapeutic use of small proteins (type 1 interferon alpha) as anti-infectives and anti-tumor agents. However, the utility of such therapy is limited by the half-life of interferon and its rapid clearance from the body. The efficacy of interferon can be improved (i.e., converted to a long acting agent) by reacting the protein with mono-methoxy polyethylene glycol to form pegylated interferon (PEG Intron®), a covalent conjugate of IFN-α_{2b} linked to a 12,000 Da PEG molecule. Pegylation occurs at any or all of numerous nucleophilic sites in the protein (the ε-amino groups of the 10 lysines, the α-amino group at the N-terminal cysteine, the imidazolyl nitrogens of the three histidines and the hydroxyl groups at the 14 serine, 10 threonines, and 5 tyrosines). Because of the numerous potential reaction sites, a heterogeneous mixture of various different modified proteins is produced.

[0007] Kinstler, O. ("Mono-N-terminal Poly(ethylene glycol)–Protein Conjugates", Adv. Drug Delivery Rev., 54, (2002) p477-485) also reports on the formation of PEG-protein conjugates. They maximize the selectivity of the PEG aldehyde conjugation to the N-terminus of an unprotected polypeptide chain by taking advantage of the differences between pK_a values of the α-amino group of the N-terminal amino acid residue and the ε amino group of the Lys residues in the peptide backbone.

[0008] Another approach is to target cysteine thiols using PEG activated with maleimides, vinyl sulfones, pyridyl disulfides, or other compounds specific for thiols, thus taking advantage of the scarcity of cysteines in proteins. Chapman, A.P., et al. ("Therapeutic Antibody Fragments with Prolonged In Vivo Half-Lives", Nat. Biotechnol, 17 (1999) p780-783) (referenced in Kinstler et al.).

[0009] The state of the art regarding polymeric drugs, polymer-drug conjugates, polymer-protein conjugates, polymeric micelles with covalently bound drugs and multi-component complexes is reviewed by Duncan, R. ("The Dawning Era of Polymer Therapeutics", Nat. Rev. Drug Discovery, 2, (May 2003) p347-360). The polymeric materials identified include PEG, HPMA, PVP, poly(ethyleneimine) (PEI),

polyamidoamines (DIVEMA), natural polymers including dextran, hyaluronic acid, chitosans and synthetic polyamino acids such as poly(L-lysine), poly(glutamic acid), poly(malic acid) and poly(aspartimides).

[0010] Hannink, J.M. ("Protein-Polymer Hybrid Amphiphiles", Angew. Chem., Int. Ed., 40, (2001) p4732-4734) discloses the irreversible association of two molecules of monobiotinylated polymers with streptavidin to form an amphiphilic protein-polymer hybrid.

[0011] Unfortunately, synthesis of the polymers with reactive end groups and separation of the excess (unreacted) polymer chains from the conjugate formed between the polymer and biomolecule is difficult and time consuming. In addition, many of these methods are not quantitative or specific and do not allow for control over the placement and number of polymer chains. To make these conjugates available, a simple and effective preparation of biomacromolecule-polymer complexes is needed.

BRIEF DESCRIPTION

[0012] The present invention generally relates to chemical polymerization initiated by, and proceeding from, a protein. In one embodiment of the invention, polymerization is initiated by a protein in the absence of additional initiation agents forming the protein-polymer conjugate. In another embodiment, polymerization is initiated in the presence of an added initiation agent that does not interact with the protein. In this case, during the polymerization process some polymer not attached to the protein is also formed. Removal of this unbound polymer results in the purified protein-polymer conjugate. The protein can be modified to contain site(s) for initiation or prepared by recombinant methods, chemical ligation, solid-phase synthesis, or otherwise with site(s) for initiation. Examples of the preparation of the conjugate are shown by the formation of a poly(N-isopropylacrylamide)-bovine serum albumin conjugate and a conjugate formed from poly(N-isopropylacrylamide) and streptavidin initiated in the presence of a solid supported initiator.

[0013] Currently, protein-polymer conjugates are prepared by synthesizing telechelic polymers (end group modified polymers) and by reacting them with proteins. This takes many steps and the products produced are difficult to purify. In a procedure incorporating features of the invention polymers are prepared by polymerizing monomers

using proteins modified with initiation sites, resulting in the formation of the protein-polymer conjugate directly. This greatly simplifies the purification procedures because the conjugates are then purified from the small monomer molecules and not from large polymer chains. It also reduces the preparation steps since there is no need for end-functionalization of the polymers, and the procedure is applicable to a wide range of proteins and hydrophilic or hydrophobic monomers. Additionally, control over the number and placement of the polymer chains on the amino acid sequence could be achieved using established protocols for site-specific modification of proteins with the initiator fragment or by expressing recombinant proteins displaying artificial amino acids containing the initiator fragment.

[0014] More specifically:

a) An initiator is added to the protein to provide the initiating site on the protein. An assortment of natural or artificial amino acids may be employed as an anchoring point for the initiator.

b) Cysteine residues naturally or artificially present on the protein are an example of anchoring points to modify the protein with the initiator; nevertheless, the invention is not restricted to the presence of cysteines on proteins. Other natural or artificial amino acids, or non-covalent interactions, can be used to conjugate the molecule that will initiate the polymerization to the protein.

c) A protein modified with the initiator is mixed with the monomer with or without added catalyst to initiate the polymerization of the monomer from the protein forming the protein-polymer conjugate in situ.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 shows a reaction scheme for modification of bovine serum albumin with an initiator fragment and polymerization from the bovine serum albumin that has been modified with an initiator.

[0016] Figure 2, shows the gel electrophoresis separation, visualized by coomassie staining, of the protein bovine serum albumin (A) and the protein-polymer conjugate formed by polymerization from the BSA-initiator (BSA-I) complex (B).

[0017] Figure 3 is a graph showing the gel permeation chromatograph trace for poly(NIPAAm) formed using modified BSA (BSA-I) as an initiator.

[0018] Figure 4 shows the chemical structure of a biotinylated initiator.

[0019] Figure 5 shows the gel electrophoresis separation of streptavidin (A) and poly(N-isopropylacrylamide)-streptavidin conjugate (B) obtained polymerizing from streptavidin previously modified with biotinylated initiators.

DETAILED DESCRIPTION

[0020] The present invention generally relates to a process for the chemical polymerization initiated from proteins to form protein-polymer conjugates, and the compositions prepared by that process. The protein can be modified with an agent(s) that can initiate a polymerization, be prepared containing such site(s) or modified by other techniques to react with the monomer. Chemical initiation and polymerization results in a protein-polymer conjugate with the initiator as a link between the protein and the polymer. Polymerization can occur with or without addition of non-interacting initiators.

[0021] As an example, the protein can be modified with an initiator either covalently bound or which forms an affinity complex with the protein. For example, bovine serum albumin (BSA) can be modified using an initiator to form a disulfide bond with a free cysteine in the BSA. However, other chemistries such as the Michael-type addition with maleimide or vinyl sulfone modified initiators can be used. As a second example, a biotinylated initiator can be synthesized and conjugated via affinity interactions to the protein streptavidin. Modification by conjugation to other amino acids, such as lysines, arginines, histidines, serines, threonines, aspartic acids, glutamic acids, or artificial amino acids, or the ends of the protein or by other affinity interactions may also be used.

[0022] Both of the initiators in the examples initiate radical polymerization, specifically atom transfer radical polymerizations (ATRP). However, other molecules that initiate radical, cationic, anionic, or metathesis polymerizations may be employed as initiators. Using this technique a large number of protein-polymer complexes can be formed quickly. Described below are complexes of albumin and streptavidin with poly(N-isopropylacrylamide) (poly(NIPAAm)). However, other polymers such as polyesters, poly(meth)acrylamides, poly(meth)acrylates, polyethers, or polystyrenes can be produced using the techniques described herein. An advantage of using a controlled polymerization technique such as ATRP is the possibility of producing polymers with narrow molecular weight distributions.

[0023] Polymer formation may be conducted using modified proteins as initiators. Compared to the current state of the art, this method has many advantages. Placement (location) and number of polymer chains is critical for properties, but difficult to control by current techniques. The process described herein readily allows the placement and number of polymer chains to be defined by the placement and number of initiating fragment(s). As an example, the albumin protein was modified at one site with the initiator. In the second example, streptavidin, which has four binding sites for biotin, was modified with the biotinylated initiator shown in Fig. 4 at three of the four sites. In addition, proteins may be prepared with defined placement and number of initiation sites by incorporation of artificial amino acids capable of initiating polymerization. For comparison, preparation of protein-polymer complexes by current techniques is more time consuming because a telechelic polymer with suitably reactive end groups must first be prepared. The polymer is then reacted with the protein and excess polymer is separated from the protein-polymer complex. Modification of proteins with small molecules is more efficient and the resulting complexes are easy to purify because the polymer is formed already attached to the protein. In addition, the technique described in this invention is flexible. For example, it is difficult and time consuming to attach a preformed hydrophobic polymer to a hydrophilic protein, whereas polymerization of a hydrophobic monomer attached to a protein is more facile.

[0024] Use of protein-polymer conjugates prepared by initiation and polymerization from the protein include, but are not limited to, use as human therapeutics, in proteomics, as protective coatings, in composite or smart materials, and in sensors. Currently there are several protein-polymer conjugates formed by prior techniques on the market or in clinical development as human therapeutics. Polymer modification of proteins provides significant clinical benefits, including increased pharmacokinetic properties. Protein-polymer conjugates can be utilized in protein array chips for proteomics applications. Conjugates, where the protein responds to stimuli and the polymer provides material stability, can be useful in protective coatings and/or clothing, and as composite materials. An example of use as a sensor material includes protein-polymer conjugates that respond to bioterrorism agents or small molecules to give a detectible color changes and/or signals. The conjugates present in bulk, organized on the surface of a carrier or formed

into micelles or liposomes, can also be used as drug delivery agents capable of selectively targeting specific tissue within the body.

[0025] The following examples, describing polymerization from albumin (Example 1) and from streptavidin (Example 2), are presented for the purpose of illustration only and not intended in anyway to limit the scope of the present invention.

Example 1. Polymerization from modified Bovine Serum Albumin (Fig. 1).

[0026] Bovine serum albumin (BSA) was modified at a single site to enable the polymerization directly from the albumin. A single free cysteine within the protein was modified to have a bromoisobutyrate functionality suitable for initiation of radical polymerization as shown in Figure 1. The initiator precursor, propyl-mercaptopyridine 2-bromoisobutyrate (1) reacts efficiently with any protein, enzyme, or antibody that, naturally or by engineering, contains a free cysteine, and thus represents a general approach. Mass spectrometry analysis of the resulting BSA initiator (BSA-I) demonstrated that the albumin was modified with no more than one molecule of initiator per BSA molecule.

[0027] Polymerization was then accomplished using a radical polymerization technique (atom transfer radical polymerization, ATRP) that can result in polymers with narrow molecular weight distributions and programmed molecular weights. BSA-poly(NIPAAm) conjugates were prepared as an example using standard ATRP polymerization conditions. Polymerization to form the conjugate is evidenced by the higher molecular weight of the conjugate compared to BSA as determined by gel electrophoresis (Figure 2). The polymer was conjugated to the protein via a disulfide bond. To demonstrate that the polymerization occurred, this bond was chemically reduced and the poly(NIPAAm) generated by polymerization from the protein was isolated. Characterization of the polymer, conducted by proton nuclear magnetic spectroscopy and infrared spectroscopy, proved the poly(NIPAAm) identity. Gel permeation chromatography confirmed that a polymer had been synthesized (Figure 3). As expected, identical polymerization conditions using unmodified BSA, did not result in formation of a polymer.

Synthesis of propyl-mercaptopyridine 2-bromoisobutyrate (1).

[0028] Hydroxypropyl-mercaptopyridine (0.50 g, 2.5 mmol), 1,3-dicyclohexylcarbodiimide (DCC, 0.516 g, 2.5 mmol) and 4-dimethylaminopyridine (DMAP, 0.031 g, 0.25 mmol) were dissolved in 20 mL of dry dichloromethane. 2-Bromo-2-methylpropionic acid (0.414 g, 2.5 mmol) was added and the reaction mixture was stirred overnight at room temperature. The reaction mixture was filtered, the solvent evaporated under vacuum and the oily residue purified by column chromatography (hexanes:ethyl acetate 60:40) to give compound 1. Yield: 53%.

Conjugation of propyl-mercaptopyridine 2-bromoisobutyrate (1) to bovine serum albumin (BSA).

[0029] 13 mg of propyl-mercaptopyridine 2-bromoisobutyrate (1) prepared as indicated above were dissolved in 1 mL of DMSO and added drop wise to a solution of BSA (2 g) in deionized, distilled water (36 mL). The mixture was incubated overnight at room temperature. After centrifuging out the insoluble residue, the product (BSA-propyl 2-bromoisobutyrate, BSA-I) was purified by dialysis and recollected after lyophilization.

Polymerization from large amounts of proteins. Example: BSA-propyl 2-bromoisobutyrate.

[0030] A Schlenk tube was charged with N-isopropylacrylamide (NIPAAm, 200 mg, 1.77 mmol) and BSA-I (400 mg). It was then evacuated and refilled with argon three times. 2 mL of degassed water were used to solubilize the monomer and the initiator. A catalyst stock solution was prepared by dissolving CuBr (8.4 mg, 0.058 mmol) and 2,2'-bipyridine (18.4 mg, 0.12 mmol) in water in oxygen-free conditions and 0.20 ml of the catalyst stock solution were added to the Schlenk tube to start the polymerization. The reaction was stopped after 2 hours by diluting with water. The product was purified by dialysis.

[0031] Polymerizations were conducted using a 0.4 g albumin sample. However, the process is readily scalable to larger quantities. Homopolymers of NIPAAm were prepared, although many different polymer compositions and architectures, including block and graft copolymers may be synthesized using this or other polymerization techniques. The process is not limited to providing bromoisobutyrate functionality or the polymerization with acrylamides and other combinations of initiators and monomers reactive therewith can be used. Proteins may be modified to have an initiation site or

prepared with an initiation site by incorporation of amino acids suitable for polymer initiation. The same general approach, using radical polymerization chemistry, can be used for the preparation of conjugates with many different proteins, enzymes and antibodies leading to functional materials.

Example 2. Polymerization from streptavidin modified with a biotinylated initiator in the presence of non-interacting initiator.

[0032] Polymerization from a protein in the presence of an added initiation agent was undertaken to demonstrate that the technique is suitable for small scale procedures, for example when only small quantities of protein are available. As an illustrative example, streptavidin was interacted with the bromoisobutyrate-modified biotin initiator shown in Fig 4. This biotin initiator binds with very high affinity to streptavidin. Polymerization of NIPAAm was initiated from the modified streptavidin with simultaneous initiation from a non-interacting bromoisobutyrate-modified solid phase resin. The non-interacting initiator does not bind to the protein, and the polymer grown from this initiator is not bound to the protein. Polymerization from streptavidin was evidenced by the higher molecular weight of the conjugate, as shown by gel electrophoresis (Fig 5).

[0033] Initiation from a very small amount of protein (<10 mg) without added non-interacting initiator may be difficult because the initiator would be very dilute. However the non-interacting initiator circumvents this by effectively increasing the number of initiation sites in the polymerization mixture. As an illustrative example, an insoluble initiator was chosen that is easily filtered away at the end of the reaction. However a soluble initiator may be used in the alternative.

Synthesis of the biotinylated initiator (Figure 4).

[0034] Biotin (1.500 g, 6.14 mmol) and N,N'-disuccinimidyl carbonate (1.573 g, 6.14 mmol) were dissolved in 25 mL of dry DMF in argon atmosphere. Triethylamine (0.745 g , 7.37 mmol) was added and the mixture stirred at room temperature for 6 hours. 2-(2-Aminoethoxy)ethanol (0.646 g, 6.14 mmol) was then added and the reaction was allowed to proceed at room temperature overnight. The DMF was then evaporated under reduced pressure, the product was dissolved in methanol and the insoluble residue was filtered out. A fraction of the pure product (400 mg, isolated after two recrystallizations from methanol/ether 1:2) was then dissolved in dry DMF (10 mL). 2-Bromo-2-

methylpropionic acid (300 mg, 1.8 mmol), N,N'-dicyclohexyl-carbodiimide (371 mg, 1.81 mmol) and DMAP (29.5 mg, 0.24 mmol) were then added and the mixture was stirred overnight at room temperature. The precipitate which formed was filtered out. After evaporating the DMF under reduced pressure, the residue was purified by column chromatography (dichloromethane:methanol = 9:1). Yield: 52%.

Synthesis of the non-interacting initiator.

[0035] A two neck round-bottomed flask was charged with ArgoGel Wang polymer beads (Aldrich, 240 mg, 0.084 - 0.108 mmol of hydroxyl groups), 2-bromo-2-methylpropionic acid (541 mg, 3.24 mmol) and DMAP (79.2 mg, 0.648 mmol) in argon atmosphere. Dry DMSO (7.5 mL) and 1,3-diisopropylcarbodiimide (500 mL, 3.24 mmol) were added and the mixture stirred at room temperature overnight. The polymer beads, which now carried the non-interacting initiator, were then filtered out and extensively washed with DMSO, water and THF and dried under high vacuum. The presence of the initiator fragment (shown in Fig. 4) on the beads was verified by IR spectroscopy (ν_{COOR} = 1731 cm^{-1}).

Modification of streptavidin with the non-covalent initiator: conjugation of the 2-bromoisobutyrate biotinylated initiator (Figure 4) to Streptavidin.

[0036] Streptavidin (4.05 mg) was dissolved in 2 mL of phosphate buffer, pH 7.0. A solution of 2-bromoisobutyrate biotinylated initiator in methanol (0.100 mL, containing 1.8 mg of biotinylated initiator) was slowly added to the streptavidin solution. The mixture was incubated at RT for 1 hour. The product was purified by dialysis and recollected after lyophilization.

Polymerization from small amounts of protein. Example: Streptavidin-2-bromoisobutyrate.

[0037] A flask was charged with the beads carrying the non-interacting initiator (10 mg, 0.0035 - 0.0045 mmol of initiating groups) and N-isopropylacrylamide (NIPAAm, 51 mg, 0.45 mmol). The system was then evacuated and refilled with argon three times and a deoxygenated solution of streptavidin (0.700 mg) modified with the biotinylated initiator in water was added. Polymerization on the beads was initiated by adding 0.050 mL of a CuBr/2,2'-bipyridine stock solution (prepared dissolving 6.4 mg of CuBr and 14.0 mg of 2,2'-bipyridine in 0.500 mL of degassed water). The reaction was stopped

after 40 minutes by opening the flask to air and separating the streptavidin solution from the beads by filtration. The protein-polymer conjugate was purified from the catalyst and the unreacted monomer by dialysis.

[0038] The above examples are merely representative of the processes described herein. Based on the teachings herein, one skilled in the art will recognize that protein-polymer conjugates can be formed from many different proteins by providing modified proteins and then reacting the modified proteins with monomers to generate the conjugate. One skilled in the art will also recognize that the methods taught herein can also be applied to the formation of polymer conjugates with a wide range of biomolecules such as enzymes or antibodies.